HISTOCHEMICAL INVESTIGATION OF THE EFFECT OF BASIC VITAL DYES UPON THE CELLS OF THE LIVER

by

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Introduction

Vital staining is one of the most frequently employed methods in the study of cell-morphology and cell function. It is well known that the stain binding capacity of the protoplasma, as well as the distribution of vital stains within the cell depends on the physiological state of the latter. Hence this method is suitable for studying the state of stimulation of the cell and/or the processes taking place in the protoplasma upon a lesion to the cell. Research work in this direction has been carried out mainly by N a s s o n o v and co-workers (10).

It is also a known fact, that, upon entering the cell, basic vital dyes cause characteristic changes. C h l o p i n (2) was the first to note that in and around the dye granules a basophile substance (krinom) accumulates. Later it was possible to demonstrate the presence of nucleic acids in the substance of krinom (5., 8., 15., 16., 21). Thus the use of basic vital stains offered the possibility of interfering under relatively physiological conditions with the nucleic acid metabolism of the cell.

These results gave rise to the idea that the effect of some basic vital dyes upon the cells should be investigated by histochemical methods, assuming that such effects would be different according to the physiological state of the cell. Combining the vital staining and histochemical methods, possibility is given for studying new aspects of the morphological manifestations of cell activity. In the first phase of our work we investigated by means of several histochemical methods, the effect of neutral red and acridine orange upon normal cells of the liver. Considering that most data found in literature refer to changes taking place after a relatively long (one day or longer) period of staining, it was necessary to thoroughly study the processes taking place within a short time as well. This paper reports the results of our work in this direction.

Material and methods

In the course of our experiments we have tested the effect of neutral red and acridine orange upon the liver of white mice. For this purpose we selected 110 mature male animals of 20—30 g. The majority of them (96 mice) were treated with neutral red. For comparison's sake we tested the effect of acridine orange upon a smaller (14 mice) group as well. Of neutral red the animals were given 25 mg of dye per 100 g body-weight, of acridine orange 14 mg of dye per 100 g body-weight. The dye was dissolved in 1 cu. cm of distilled water and administered intra-peritoneally in the form of a single injection. The control animals were given simultaneously 1 cu. cm of distilled water.

The mice were killed after 1., 2., 4., 6., 8., 16., 24 hours respectively counted from the administration of dye or distilled water. In some cases we have tested the effect of the stain 2 days and/or 1, 2 and 3 weeks after the administration.

The animals were killed by decapitation, then small pieces were cut out of the liver and fixed in neutral formalin containing sucrose (4), in the Carnoy and Helly solutions as well as in 1% osmium acid. In addition we determined the amount of neutral red bound by the cells of the liver in 25 animals. For this purpose we extracted by means of acidic alcohol the dyestuff from the pieces of liver and determined the extinction of the obtained solution by means of the Pulfrich photometer (10).

With the histochemical methods applied in the course of these experiments we tested the presence of nucleic acid, proteins and carbo-hydrates, as well as of acid phosphatase and lipoids in the liver cells. For the joint detection of ribonucleic acid (RNA) and desoxyribonucleic acid (DNA) we applied 0,1% solution of toluidine blue (pH 3,8), for the detection of DNA Feulgen's reaction. Part of the sections were treated with ribonuclease before staining. For detecting the proteins we have employed the Millon and ninhydrin-Schiff reaction, and the staining with bromphenol-blue (13).

For testing the presence of carbo-hydrates we have applied the PAS reaction. Prior to setting off the PAS reaction glycogene was removed from some sections by saliva digestion. The detection of acid phosphatase was effected with Gömöri's method. For controlling specificity in some sections we inactivated the ferment by hot-water or Lugol-solution treatment before testing. The lipoids were stained by Sudan black and Sudan red 7/B solutions respectively. The study of mitochondria was carried out in the slices fixed in osmium acid, by means of Heidenhain's iron hematoxylin staining.

Results

On opening the abdominal cavity of the animals treated with neutral red, the intense red colouration of the liver is well visible even to the naked eye. The dye content of the liver varies according to the different periods following administration. The data are contained in Table 1.

It appears from the results shown in the Table, that the amount of neutral red in the liver cells is largest in the first hours following administration and is reduced at an increasing rate later on.

Table 1.

Changes in the neutral-red content of the liver cells of mice measured after different periods following the administration of the dye.

Time elapsed after the administration of the dye	Extinction/g. dry weight
1 hour	35,67 ± 4,5
4 hours	23,89 ± 2,9
8 hours	16,78 ± 2,9
16 hours	6,41 ± 2,9
24 hours	1,29 ± 0,7

Before describing the morphological changes caused by neutral red a brief summary is given of the results obtained upon examination of the control animals. In the liver cells of the control animals a large quantity of RNA-containing basophile substance was noted in the form of irregular clumps dispersed at random in the cytoplasm. The nucleus, particularly the membrane and the chromatin substance are easily stained with toluidine-blue and were found to be Feulgen-positive.

Following removal of the glycogene, small PAS-positive grains are seen in the cytoplasm of the liver cells along the bile-canaliculi. Granules colouring in the same way, but of larger dimensions, can be observed also in the Kupffer-cells. Acid phosphatase activity can be detected in the granules of the liver cells and the Kupffer cells. (Fig. 3.) Considering their number, shape and position, these are identical with the afore-mentioned PAS-positive granules and can evidently be identified with the lysosomes described by several authors.

Following detection of the proteins, colouration can be observed both in the cytoplasm and in the cell nucleus. Pale diffuse sudanophilia can be demonstrated in the liver cells of the control animals, while drops of fat can seldom be found in the cytoplasm.

The mitochondria are grain-like or rod shaped. The granular shape is characteristic rather of the centrilobular cells.

Neutral red causes characteristic morphological changes in the liver cells of mice. In the slices of liver fixed in the first hours following administration, the stain cannot be seen, probably because the fixatives destroy the developing dye vacuoles. However, 2–3 hours after administration the neutral red will get bound to the substance of the cytoplasm and the dye granules formed in this way will already stand. On sections made of such liver the pale, yellowish red dye granules can be well perceived after paraffine is removed.

2–3 hours after the administration of the neutral red small granules containing RNA appear in the liver cells of the animals and can be readily detected with toluidine blue. These granules can be seen in the largest number in the centrilobular cells, but appear also in the perilobular cells. The difference between the centri and perilobular cells increases later on. In the former, number and dimension of the basophile grains increase, more and more sub-

stance containing RNA accumulating in them; at the same time, the basophilia of the cytoplasm gradually diminishes (Fig. 1). In the perilobular cells the development of the basophile grains can also be observed, yet their number and dimensions will always be considerably smaller than in the centrilobular cells. The peak of the process is reached 6 to 8 hours after the administration of the dye. The basophile grains are the largest at this time, then they will gradually disappear from the cells, first from the cytoplasm of the perilobular cells. In the 16th hour basophile granules are usually discernible in the cells near the vena centralis only, while in the 24th hour these disappeare too.

Fig. 1. Mouse, centrilobular cells of the liver, 6 hours following administration of neutral red. Helly, toluidine-blue, 9 × 60. Basophile granules in the cytoplasm of the cells

Fig. 2. Mouse, centrilobular cells of the liver, 24 hours following administration of neutral red. Helly, toluidine-blue, 9 × 60. Cells of diffuse staining

If the distribution of RNA-containing basophile grains in the cell is examined, it appears in most of the cases that they are placed along the bile-canaliculi, but they can often be found irregularly dispersed or around the nuclei as well. A check with considerable magnification will reveal that the basophile substance appears first on the surface of those dye granules which contain neutral red, and surrounds them like a cap.

As a consequence of the development and subsequent elimination of the basophile grains, the basophilia of the cytoplasm will considerably be decreased 24 hours after the administration of the stain. The difference between the peri — and centrilobular cells will be significant even then. In the former, the remnants of the basophile substance of the cytoplasm can be seen in the form

of clumps situated mostly next to the nucleus. However, in the cytoplasm of the centrilobular cells the remaining basophile substance is mostly distributed in a diffuse state, in some cases, a threaded structure being observable in them (Fig. 2). The examination of the liver of animals killed at a later date reveals that the organ begins to resume the original state in about a week's time, while in two weeks' time distribution of the RNA will approach the normal picture.

The presence of neutral red will cause changes also in the nucleus. Examining the structure of the nucleus it appears that it does not differ from the normal during the first 6 to 8 hours of staining. After a longer (16 to 24 hours) action of the dye, however, signs of lesion appear in the cells around the vena centralis. The nuclear membrane thickens and chromatine precipitates. Later on, the breaking into pieces of the nucleus can be observed as well. The number and dimensions of the nucleoli will be normal in the beginning, but in the 24-hour substance, they are considerably smaller than in the control animals. In the liver of the animals treated with neutral red, abnormal mitosis will be frequent even 2 to 3 weeks following administration. Cell nuclei of gigantic dimensions will frequently occur. This shows that neutral red seriously damages the cells of the liver.

It should be added that basophile grains will develop in the Kupffer cells as well; they will be, however, considerably smaller than in the parenchyma cells.

The various fixatives will not preserve in the same way the basophile grains developing under the effect of the stain. Most suitable for this purpose are formaline and the Helly solution. They will keep also in Carnoy but in a more unstable condition and will easily dissolve in the course of processing. If, however, the sections fixed in Carnoy are placed onto the slide in a dry state and coated by celloidine prior to staining, conditions for studying the basophile grains will be very favourable.

Following the dissolution of glycogene, PAS-positive grains can be detected in the liver cells of animals treated with neutral red. As regards their distribution, number and dimensions, these granules are identical with the basophile grains containing RNA. These grains containing PAS-positive substance take up their position in the same place as the lysosomes and cannot even be distinguished from the latter in the first hours following the administration of neutral red. Later on (after 4—6 hours) their dimensions will gradually increase, they will accumulate mainly in the centrilobular cells, then again later (16 to 24 hours) they will gradually disappear form the cells. However, even after their disappearance a few small lysosomes will be retained in the cells. From the aforesaid it follows that, in addition to RNA, the basophile granules contain PAS-positive substance as well and that these two components behave in the same way. It has also been proved in the course of our experiments that for detecting the PAS-positive substance of the granules various fixatives are not equally suitable. Following formaline and Helly fixation, it can be readily shown, but not after Carnoy.

The activity of the acid phosphatase does not substantially change in case of a dye effect of short duration (1 to 4 hours). Later, however, in the cells around the vena centralis, where the neutral red accumulates to an increased extent, the activity of the acid phosphatase diminishes, and ceases

completely in many cases while the non-specific reaction of the nuclei increase at the same time (Fig. 4). In the perilobular cells, the ferment can always be detected.

In case of short stain (1 to 4 hours), the distribution of the lipoids agrees with that of the control. Later on fat vacuoles appear in the centrilobular cells. After 16 to 24 hours, also most of the peripheral cells will contain many lipoid drops. In can often be observed in the 16 hour material that sudanophile substance covers the surface of the dye grains, like a cap, in the same way as can be seen in the case of detecting the PAS reaction or RNA.

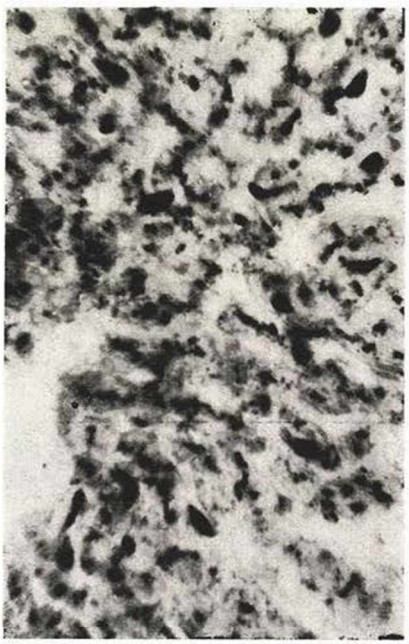

Fig. 3. Mouse, perilobular cells of the liver, control. Formol, acid phosphatase, 9 × 40

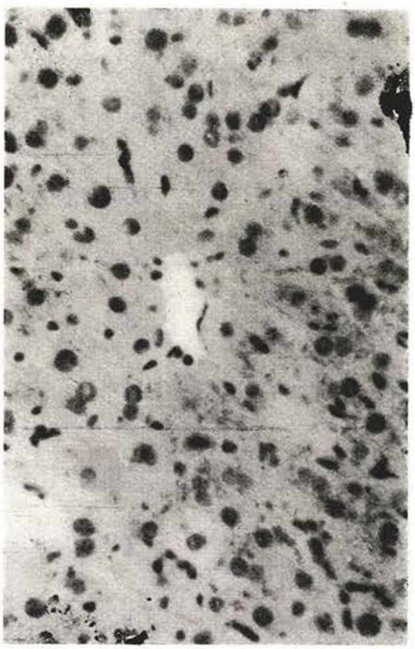

Fig. 4. Mouse, centrilobular cells of the liver, 24 hours following administration of neutral red. Formol, acid phosphatase, 9 × 40

According to the results obtained through protein reactions, the basophile granules developing under the effect of neutral red do not contain any protein or, if so, only a minimum quantity.

Testing the mitochondria we have not found any substantial morphological changes. In the case of longer (24 hours) neutral red action, the mitochondria could often be observed on the edges of the cells.

According to our experience acridine orange has an effect similar to that of neutral red. In this case, too, the development of the characteristic basophile, PAS-positive granules can be well observed in the second or third hour following the administration of the dye. Their behaviour is the same as that of the granules developing under the action of neutral red.

Discussion

As it appears from our results, neutral red and acridine orange elicit a characteristic response in the liver cells of mice. The most striking change can be observed in the distribution of the RNA-containing substance of the cytoplasm and the intensity of its staining. It was discovered by Chlopin (2) that under the action of neutral red, a basophile substance, the so-called Krinom, appears in the form of granules in the cytoplasm of the cells, and that the granules accumulate in the neutral red grains and around them. A certain time is required for their formation. The krinom substance can well be shown also in the fixed cells. According to Chlopin (2), its formation can be observed in several types of cell, thus in the liver cells as well. This is supported also by Weatherford's findings (18) who observed krinom formation in the liver cells of the frog after a neutral red staining of several days. According to Chlopin (2) the substance of krinom is a protein that can be painted by basic stains.

Later research has been directed at the exploration of the mechanism of the process and a more accurate determination of the substance of krinom respectively. Kamnev (5) was the first to show in the intestinal epithelial cells of the frog that DNA can occasionally be observed in the granules formed under the action of neutral red. Kedrowski (6, 7, 8) has established the presence of the so-called acid colloid (RNA). He claimed that the basophile substance of the krinom grains is present in the cell already prior to the intrusion of the vital stain and is distributed diffusely in the cytoplasm. Under the action of the stain it precipitates in grain form. According to Zeiger and Schmidt (21) as well as Schmidt (15, 16) krinom containing RNA and/or DNA can be discerned in the cytoplasm of cells treated with vital stains. The occurrence of RNA and DNA may vary according to the type of cell.

The accumulation of basophile krinom granules in the cytoplasm can be observed also under the action of acridine orange (15, 16, 19, 20, 21).

Our data concerning the RNA content of the krinom are in good agreement with those found in the literature referred to above.

The presence of nucleic acids in basophile granules has been investigated in detail, but few data are available concerning the presence of substances of other nature. According to our investigations, the krinom granules formed in the liver cells are characterised from the histochemical point of view not only by RNA content but by PAS-positivity as well. The presence of a PAS-positive substance in granules formed in the ascite cells of mice under the action of acridine orange is mentioned briefly by Wittekind (20). However, according to Zeiger and Schmidt (21), krinom does not contain any PAS-positive substance. This contradiction is probably due to the fact that the PAS-positive substance is of an unstable nature and can be detected only in cells fixed in formaline or in Helly solution.

The references in literature concerning the protein content of krinom are inconsistent. According to some authors (2, 19) it contains protein. It should be noted, however, that no specific methods suitable for the detection of protein have been employed in this work. According to our findings, the substance

of krinom contains no protein, or a minimum quantity at most. This agrees with the observations made by Kedrowski (8). According to the findings of Zeiger and Schmidt (21) as well as Schmidt (15) in the krinom grains containing DNA some kind of substance, well visible by the phase contrast microscope, is retained after the removal of the DNA and this substance contains protein. Such substance cannot be detected in the RNA-containing krinom granules after the removal of RNA. These data are confirmed also by the electron-microscopic investigations of Schmidt (16).

Analysing the histochemical properties of the krinom granules, the presence of lipoids should be mentioned. In our experiments, the sudanophile substance appeared on the surface of the stain granules after a neutral red action of relatively long duration (16 hours). The presence of lipoids has been observed under similar conditions by Schmidt (15) as well. According to Ogawa and co-workers (12) the stain granules developing under the action of neutral red in the fibroblasts contain lipoproteids as well as acid and alkaline phosphatase and lipase. According to our findings the activity of acid phosphatase will be highly reduced, or not detectable at all, in the centrilobular cells, where neutral red accumulates in large quantities under the action of staining of longer duration.

The references in literature concerning the formation of krinom are related to changes occurring under the action of relatively long staining, taking several days, or stain injections repeated several times. As appears from our results, the accumulation of the basophile substance in the dye granules can be observed in the liver cells of white mice as early as in the second or third hour following the administration of the stain. It is noteworthy that the centrilobular and/or perilobular cells behave differently. In the former, the size of the krinom grains gradually increases, most of the RNA content of the cell becoming concentrated in them. From the perilobular cells, the stain is eliminated more quickly, the krinom granules are invariably smaller and disappear more rapidly, than from the centrilobular cells. This difference is probably due to the fact that the blood supply of the perilobular cells is better than that of the centrilobular cells and that their metabolic processes are more intensive as well. The circumstance should also be mentioned that, having reached a certain concentration, the stain probably paralyses the mechanism of elimination in the cell. This is supported by the data of Zeiger and Wiede (22) who observed that a small amount of acridine orange will easily be eliminated from the liver cells of the frog into the bile capillaries, but, if entering the cells in considerable quantities, elimination will be blocked. Probably a similar process is taking place under the action of neutral red in the centrilobular cells of the liver of mice. This may serve as an explanation of the fact that although the amount of the stain begins to gradually diminish in the whole of the liver within a few hours after administration, it still remains in the centrilobular cells for a longer time.

Assessing the results, the problem presenting itself in this: which are the parts of cytoplasm in which the formation of dye granules and the krinom respectively, takes place. As is well known, in studying the vital staining of liver cells, authors generally state that the dye accumulates in the Golgi zone (2, 9, 14, 18).

As a result of work carried out by de Duve, Novikoff (3, 11) and others, a new group of the cytoplasm particles, the lysosomes, have been discovered and investigated. They are to be found mainly along the bile capillaries, contain much acid hydrolitic enzymes (including acid phosphatase), as well as phospholipides and PAS-positive substance. Electronmicroscopic examinations show that they are surrounded by an unit membrane. Among others, the lysosomes play an important part in the accumulation and decomposition of foreign substances entering the cell. Thus the question arises whether or not the neutral red entering the cells accumulates in the lysosomes. Such possibilities are pointed to by Brachet (1). According to data by de Duve (3) it is possible to establish by biochemical methods, that part of the neutral red entering the liver cells is localised in the lysosoma fractions. The results of the electron-microscopic examinations also support the possibility that the vital stains may accumulate in the lysosomes (17). In our experiments, the presence of lysosomes in the liver cells of the control animals was easily detected by means of acid phosphatase and PAS reactions respectively. Shape, number and distribution of the granules containing neutral red, as well as their positive PAS reaction agreed with those of the lysosomes. This fact admits of the conclusion that neutral red accumulates in the lysosomes of the liver cells. Under its action these will be transformed substantially, increase in dimension, RNA accumulating in them, while their acid phosphatase activity will be diminished.

Summary

In our experiments, we have investigated by some histochemical methods the effect of neutral red and acridine orange upon the liver cells of mice. We have established that two hours after their administration, a basophile substance (krinom) of RNA content accumulates in the stain granules and around them. The dimensions of the granules and their RNA content increase gradually in the centrilobular cells and become the largest in approximately 6 to 8 hours following administration of the stain, where-upon a gradual decrease takes place and in from 16 to 24 hours after staining the dye together with the krinom substance disappears from the cells. As a consequence, this process results in a substantial reduction of the basophilia of the cytoplasm and signs of lesions can be observed in the cells. The substance of krinom is characterised by its RNA content as well as its positive PAS reaction.

On the basis of these results we came to the conclusion that neutral red and/or acridine orange accumulate in the lysosomes of the liver cells. Under the action of the stain their acid phosphatase activity will be reduced to cease altogether and, as a consequence of krinom formation, a RNA containing substance appears in them simultaneously.

РЕЗЮМЕ

В ходе работы при помощи гистохимических методов окраски было изучено влияние нейтрального красного и акридинового оранжевого на печеночные клетки белых мышей. Авторы установили, что через два часа после введения красителей, они отлагаются в цитоплазме печеночных клеток в виде гранул, в которых накопляется базофильное вещество, содержащее РНК (так называемый кринoм Хлопина). Размеры гранул и содер-

жание РНК в клетках, находящихся в центральной части печеночной дольки постепенно увеличиваются и достигают мксимума в 6 – 8 ч. после введения красителя, потом наблюдается постепенное снижение и через 16 – 24 ч. после инъекции гранулы красителя вместе с накопленной в них РНК исчезают из цитоплазмы клеток. В итоге этого, базофильность цитоплазмы значительно снижается и в клетках отмечаются признаки повреждения. Вещество кринома характеризуется содержанием РНК и материала, дающего положительную ШИК-рекацию.

На основании полученных данных можно сделать вывод о том, что нейтральный красный и акридиновый оранжевый накопляется в лизосомах печеночных клеток. Под влиянинм этих красителей активность кислой фосфатазы в лизосомах постепенно снижается и наконец исчезает. Одновременно с этим, в результате образования кринома в лизосомах появляется РНК.

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